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Article

Deacetylcephalosporin C Production in *Penicillium chrysogenum* by Expression of the Isopenicillin N Epimerization, Ring Expansion, and Acetylation Genes

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SUMMARY

Penicillium chrysogenum npe6 **lacking isopenicillin N acyltransferase activity is an excellent host for production of different β-lactam antibiotics. We have constructed *P. chrysogenum* strains expressing *cefD1*, *cefD2*, *cefEF*, and *cefG* genes cloned from *Acremonium chrysogenum*. Northern analysis revealed that the four genes were expressed in *P. chrysogenum*. The recombinant strains TA64, TA71, and TA98 secreted significant amounts of deacetylcephalosporin C, but cephalosporin C was not detected in the culture broths. DAC-acetyltransferase activity was found in all transformants containing the *cefG* gene. HPLC analysis of cell extracts showed that transformant TA64, TA71, and TA98 accumulate intracellularly deacetylcephalosporin C and, in the last strain (TA98), also cephalosporin C. Mass spectra analysis confirmed that transformant TA98 synthesize true deacetylcephalosporin C and cephalosporin C. Even when accumulated intracellularly, cephalosporin C was not found in the culture broth.**

INTRODUCTION

Since the introduction of submerged cultures of *Penicillium chrysogenum*, the β-lactams fermentation technology has been extensively developed [1, 2]. *P. chrysogenum* NRRL1951, the wild-type strain isolated in Peoria (Illinois), produced about 70 mg/l of penicillin, whereas, as the result of strain-development programs and fermentation-technology improvement, the strains used by the penicillin-producing companies synthesize more than 50 g/l of penicillin [2, 3]. Thus, *P. chrysogenum* is likely to be a good host microorganism for the production of β-lactams other than penicillin, by using genetically modified strains able to express genes for the biosynthesis of different β-lactams. Indeed, a process for industrial production of cephalosporin intermediates in *P. chrysogenum* has been developed by using the bacterial genes involved in cephalosporin biosynthesis (US patent 2002037547; European patents EP0532341 and EP0540210).

Cephalosporins are produced industrially by the filamentous fungus *Acremonium chrysogenum* [4]. Other ascomycetes including species of the genera *Paecilomyces* [5], *Scopalariopsis*, *Diheterospora*, *Spiroidium* [6], and the wood-inhabiting marine fungus *Kallichroma tethys* [7], contain genes for biosynthesis of cephem-type antibiotics [7–9] and may provide genes to obtain modified cephem antibiotics. The biosynthesis of cephalosporins in *A. chrysogenum* (reviewed by [10–13]) begins with the formation of the tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) by the nonribosomal ACV peptide synthetase (encoded by the *pcbAB* gene [14]) (Figure 1). The ACV tripeptide is cycled to isopenicillin N (IPN) by the IPN-synthase (a Fe[II] and α-ketoglutarate-dependent dioxygenase) encoded by *pcbC* [15]. IPN is later converted into penicillin N (PenN) by an epimerase system that has remained uncharacterized for decades. Recently, we reported that the conversion of IPN into PenN is carried out by a novel epimerization system involving the combined action of an isopenicillin N-CoA (IPN-CoA) synthetase encoded by the *cefD1* gene and an IPN-CoA epimerase encoded by the *cefD2* gene [16]. This epimerization system is similar to epimerases involved in chiral inversion systems in other eukaryotic cells [17–19], but it is entirely different from the known pyridoxal phosphate-dependent IPN epimerase involved in the biosynthesis of bacterial β-lactam antibiotics [9, 20, 21, 22, 23]. After the epimerization step, a bifunctional enzyme with expandase and hydroxylase activities (encoded by the *cefEF* gene [24]) converts penicillin N into deacetoxycephalosporin C and deacetylcephalosporin C (DAC); finally, DAC is converted into cephalosporin C by the DAC-acetyltransferase encoded by the *cefG* gene [25, 26]. Expression of the *cefG* gene is limiting in wild-type *A. chrysogenum* strains [4, 27]. The first two genes of the pathway, *pcbAB-pcbC*, are linked forming the "early" gene cluster located in chromosome VII, whereas the *cefEF* and *cefG* genes forming the so-called "late" gene cephalosporin cluster are located in chromosome I [11]. The *cefD1-cefD2* genes encoding the IPN epimerization system are linked to *pcbAB-pcbC* in the "early" gene cluster [9, 16].

Due to its genetic characteristics, that result in the biosynthesis of large amounts of the ACV tripeptide and IPN, good sporulation, and growth kinetics, *P. chrysogenum* is

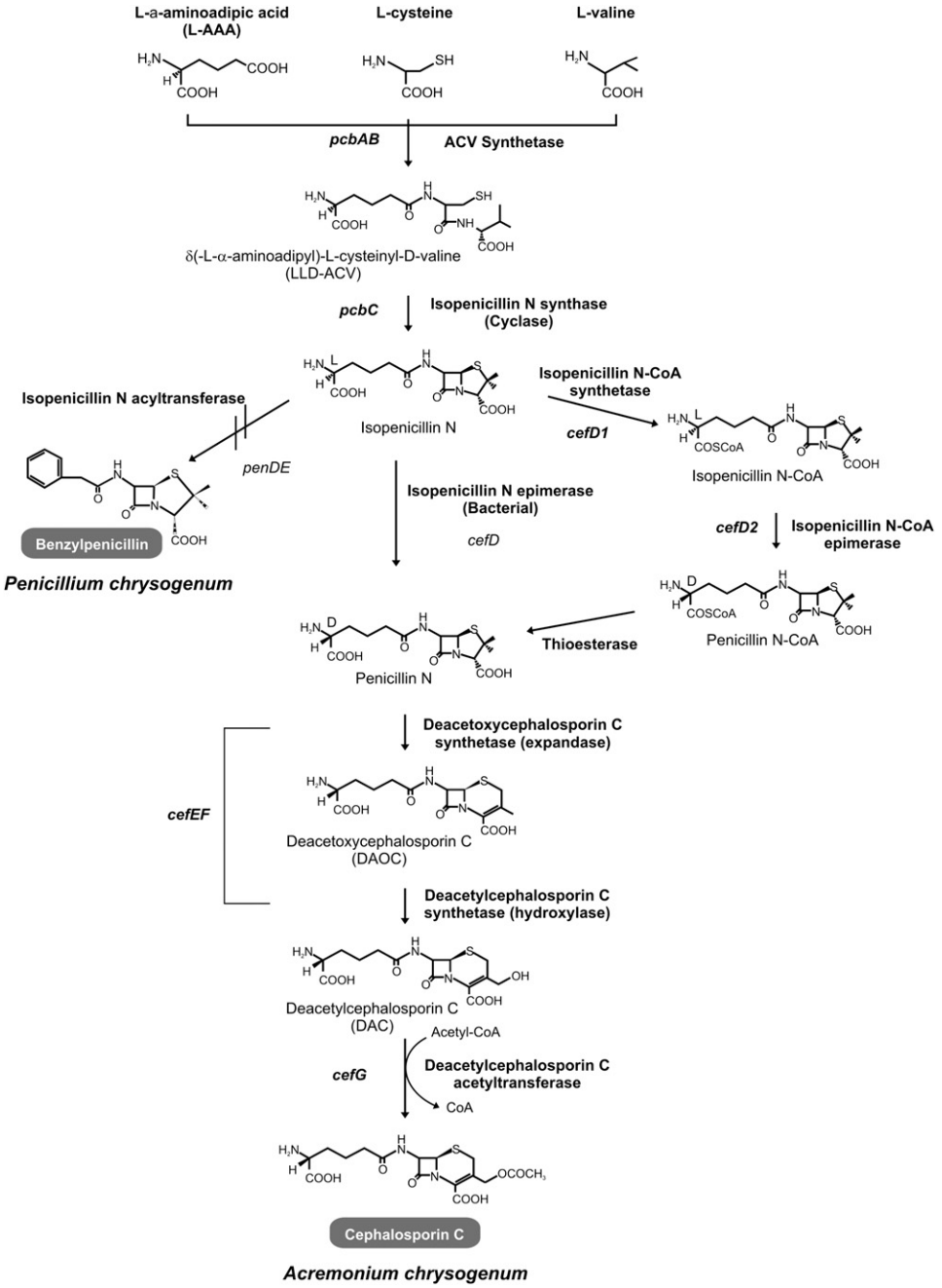

Figure 1. Biosynthetic Pathways of Cephalosporin C in *A. chrysogenum* and Benzylpenicillin in *P. chrysogenum*
Note that *P. chrysogenum npe6* is a nonproducer mutant altered in the *penDE* gene lacking isopenicillin N acyltransferase (symbol ≠).

likely to be an excellent host strain for production of either classical cephalosporins or novel β-lactam compounds. Since all the genes required for cephalosporin biosynthesis are now known and available in our laboratory, it was of great interest to study their expression in the heterologous host *P. chrysogenum* to analyze the ability of this fungus to produce and secrete cephem-type compounds. For this purpose, a *P. chrysogenum npe6* strain lacking IPN acyltransferase activity due to a point mutation in the *penDE* gene [28] that accumulates IPN was selected as host for the metabolic engineering studies.

In this article, we described for the first time, to our knowledge, the synthesis of deacetylcephalosporin and cephalosporin C in *P. chrysogenum* by using the genetic information needed for the biosynthesis of cephalosporins from *A. chrysogenum*. The results obtained in our work indicate that *P. chrysogenum* can be used as heterologous host for the production of deacetylcephalosporin C.

RESULTS

Integration in the Host Strain of the "Late" Cluster *cefEF* and *cefG* Genes of *A. chrysogenum*

In order to construct a cephalosporin-producing *P. chrysogenum* strain, the last two genes of the cephalosporin pathway *cefEF* and *cefG* were introduced into *P. chrysogenum npe*6 *pyrG*, a mutant lacking isopenicillin N acyltransferase activity [28]. Protoplasts of *P. chrysogenum npe6 pyrG* were transformed with the integrative plasmid p43EFG (see Figure S1A in the Supplemental Data available with this article online) that bears the *cefEF* gene with its own promoter and the cDNA of *cefG* under the control of the *P. chrysogenum pcbC* promoter (this promoter was preferred because the *cefG* gene is expressed very poorly from its native promoter [27]). This vector contains also the phleomycin resistance marker used for transformant selection.

*P. chrysogenum npe*6 *pyrG* transformants with plasmid p43EFG were analyzed by Southern blot hybridization to select one strain with nonreorganized copies of the exogenous *cefEF* and *cefG* genes. Results showed that transformant TA2 have a 2.1 kb SalI band hybridizing with a 543 bp *Nco*I/KpnI *cefG* probe that corresponds to an unreorganized copy of the *cefG* gene. Similarly, the genomic DNA of transformant TA2 was hybridized with a 500 bp SalI *cefEF* probe; this transformant showed a 2.7 kb *Xba*I hybridization band that corresponds to an intact copy of the *cefEF* gene (Figure S1B). Thus, *P. chrysogenum* transformant TA2 have integrated both *cefEF* and *cefG* exogenous genes.

Integration of *cefD1* and *cefD2* Genes Encoding the Epimerase System of *A. chrysogenum*

P. chrysogenum TA2 lacks the genes encoding the fungal isopenicillin N epimerization system [16] to be able to produce cephalosporins. To develop a *P. chrysogenum* strain able to express these genes, the *P. chrysogenum* TA2 strain was cotransformed with plasmids pCD1+2 (Figure S2A) and pBG (as a helper for transformant selection). pCD1+2 contains the *Acremonium cefD1* (encoding the IPN-CoA synthetase) and *cefD2* (encoding the IPN-CoA epimerase) genes under the control of their native promoters, and plasmid pBG bears the *pyrG* gene of *P. chrysogenum* that complements the uracil auxotrophy.

The transformants selected by complementation of the auxotrophy were analyzed by Southern blot to confirm the presence of unreorganized copies of the *cefD1* and *cefD2* genes. Results showed that three prototrophic transformants TA64, TA71, and TA98 contained unreorganized copies of the *cefD1* and *cefD2* genes (Figure S2B) and were selected for further studies.

Production of Penicillins and Cephem Compounds in *P. chrysogenum* TA64, TA71, and TA98

P. chrysogenum TA64, TA71, and TA98 have, therefore, the genetic information necessary for the biosynthesis of β-lactam compounds not synthesized in nature by this fungus; these transformants should be able to synthesize penicillin N and also different cephem compounds like deacetylcephalosporin C (DAC) and cephalosporin C. The production of these compounds was quantified in *P. chrysogenum* TA64, TA71, and TA98 cultures in CP medium without phenylacetic acid (phenylacetic acid is used in *P. chrysogenum* for the biosynthesis of benzylpenicillin, which can not be synthesized by the recombinant strains because they have an inactive IPN acyltransferase and is toxic for mutants lacking this enzyme). In these studies, *P. chrysogenum* Wis 54-1255 (the parental strain of Wis 54-1255 *npe6*), *P. chrysogenum npe6*, and *P. chrysogenum* TA2 were used as control strains.

Results showed (Figure 2) that *P. chrysogenum npe6* and TA2 synthesized a β-lactam (later identified as isopenicillin N), while transformants TA64, TA71, and TA98 produced a higher level of total β-lactams. The production of total β-lactams in the three TA64 to TA98 transformants started later than that of penicillin in Wis 54-1255, but the final total β-lactam concentrations were similar in the three transformants than in the Wis 54-1255 control strain in CP medium without phenylacetic acid. Bioassays of culture-broth samples with and without penicillinase treatment to remove the penicillin intermediates showed that only transformants TA64, TA71, and TA98 (containing all genes of the cephalosporin pathway) were able to produce cephalosporins, while in strains Wis 54-1255 and TA2, no detectable amounts of cephalosporins were found. A comparison of the cephalosporins (Figure 2B) and total β-lactam yields (Figure 2A) indicated that about 75% of the total β-lactams produced were penicillinase-resistant cephem-type compounds.

Culture Broths of *P. chrysogenum* TA64, TA71, and TA98 Contain Isopenicillin N, Penicillin N, Deacetylcephalosporin C, But No Cephalosporin C

In order to determine which β-lactam compounds were synthesized, the culture broth of *P. chrysogenum* TA2 and *P. chrysogenum* TA98 were analyzed by HPLC. Results showed (Figure 3) that *P. chrysogenum* TA2 (lacking the epimerization genes) did not produce penicillin N or cephalosporins and accumulated isopenicillin N, as expected. HPLC resolution of hydrophilic penicillins showed that *P. chrysogenum* TA98 produce isopenicillin N and also penicillin N, demonstrating, to the best of our knowledge, for the first time, the formation of penicillin N in *P. chrysogenum* by using the *Acremonium* epimerase genes. Finally, HPLC analysis of the cephem compounds showed that *P. chrysogenum* TA98 produce deacetylcephalosporin C at different cultivation times, while cephalosporin C was not detected in significant amounts in the culture broths. The same results were found in *P. chrysogenum* TA64 and TA71.

The *cefG* Gene and All the Other Genes of the Cephalosporin Pathway Are Expressed in *P. chrysogenum*

As indicated above, the *cefG* gene was coupled to the *P. chrysogenum pcbC* promoter and, therefore, should be expressed efficiently in the homologous host. To confirm

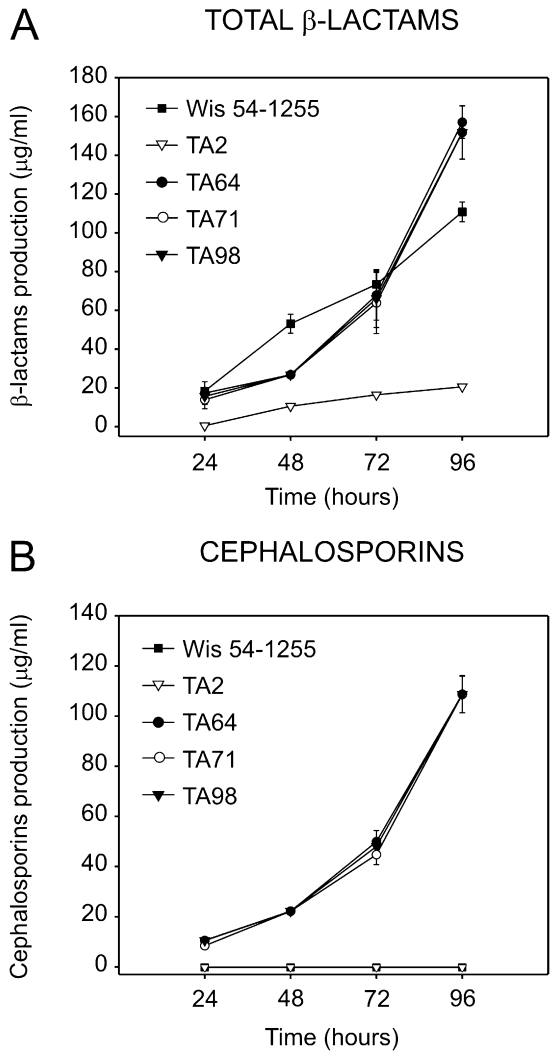

Figure 2. Production of Total β-Lactams and Cephalosporins in CP Medium Cultures without Phenylacetic Acid of Transformants TA64, TA71, TA98, TA2, and the *npe6 pyrG* Parental Strain *P. chrysogenum* Wis 54-1255

(A) Total β-lactams; (B) cephalosporins. Samples were taken every 24 hr to determine the antibiotic production. The cultures were performed in duplicate flasks in parallel and were repeated three times. Bioassays were made with *E. coli* ESS2231 without (β-lactams) and with (for cephalosporins) treatment with penicillinase (see text). Vertical bars indicate standard deviation values. Note that the Wis 54-1255 and TA2 symbols in (B) overlap completely.

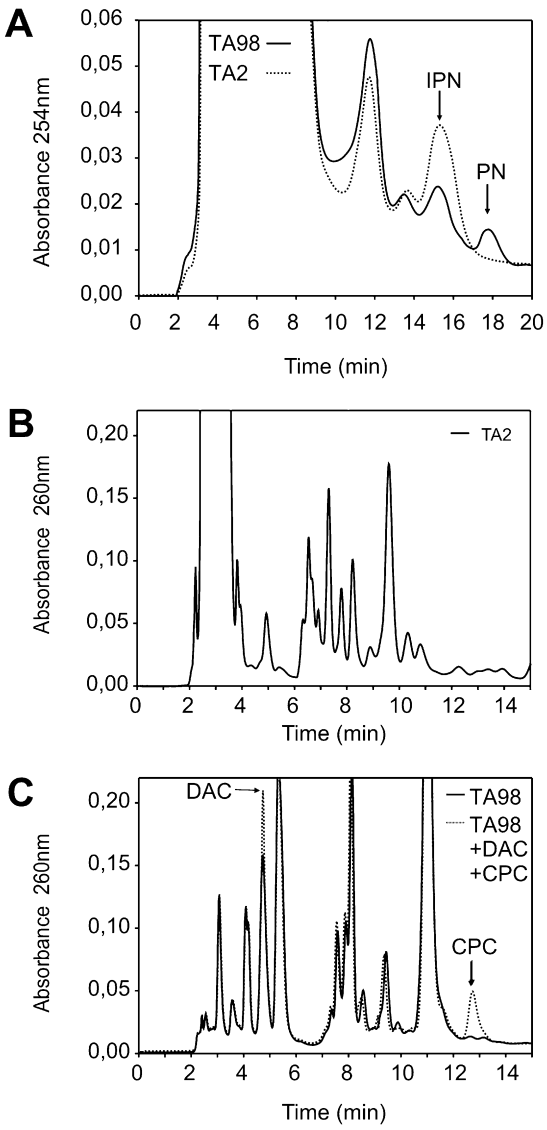

Figure 3. Identification of Intermediates of Cephalosporin Biosynthesis in Cultures of *P. chrysogenum* Transformants

(A) HPLC resolution of IPN and PenN as GITC derivatives in supernatants of *P. chrysogenum* TA2 and TA98 grown in CP medium for 72 hr. Note that there is no formation of PenN and an accumulation of IPN in the TA2 transformant lacking the epimerization system, whereas PenN is formed in transformants TA98 bearing the *cefD1* and *cefD2* genes. (B) HPLC chromatograms of underivatized culture supernatants showing the lack of DAC and cephalosporin C in the supernatants of transformed TA2 grown in CP medium without phenylacetic acid for 96 hr. (C) HPLC chromatograms showing the presence of DAC and the absence of cephalosporin C (arrows) in the supernatants of TA98 grown in CP medium for 96 hr. The DAC and cephalosporin C peaks were identified by coelution with a mixture of pure DAC and cephalosporin C (dotted line).

that the absence of cephalosporin C in the culture broths of the transformants is not due to a lack of transcription of the *cefG* gene, the transcript levels of *cefD1*, *cefD2*, *cefEF*, and *cefG* were compared by northern analysis in *P. chrysogenum* TA98 and in the high cephalosporin producer *A. chrysogenum* C10. As shown in Figure 4, all four genes were expressed in *P. chrysogenum* TA98 at levels similar or even higher (e.g., *cefD2*) than those in *A. chrysogenum* C10. The *cefD2* gene that encodes the isopenicillin N-CoA epimerase and is known to be limiting for cephalosporin biosynthesis in *A. chrysogenum* C10 [29] was expressed with very high intensity in *P. chrysogenum* (Figure 4). The *cefG* gene converting DAC into cephalosporin C was expressed to a similar extent in both *A. chrysogenum* (from its native promoter) and *P. chrysogenum* TA98 (from the homologous *pcbC* promoter) (see Discussion).

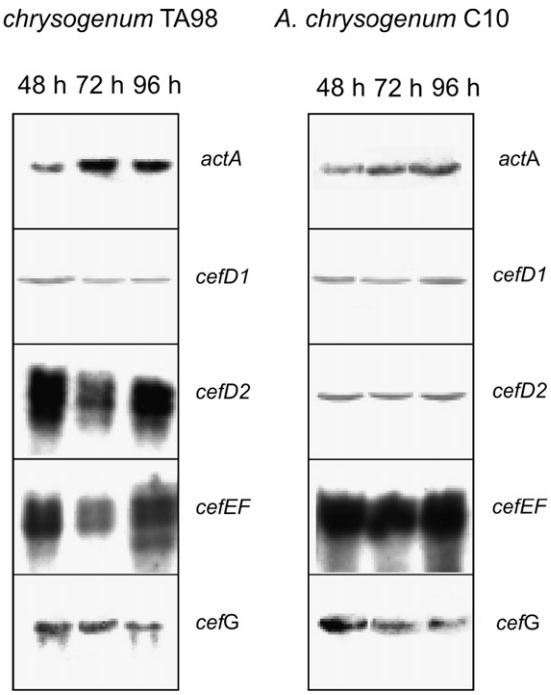

Figure 4. Northern Blots of the *cefD1*, *cefD2*, *cefEF*, and *cefG* Genes in *P. chrysogenum* TA98 Compared with the Expression of the Same Genes in the Improved Cephalosporin Producer Strain *Acremonium chrysogenum* C10

Expression of the γ-actin gene (*actA*) was used as control; an *Nco*I-*Kpn*I 0.8 kb fragment of the *A. nidulans actin* gene was used as probe. Note the high expression of the *cefD2* gene (isopenicillin N-CoA epimerase) in *P. chrysogenum*. The *cefG* gene in *A. chrysogenum* C10 is expressed from its native promoter, whereas in *P. chrysogenum*, this promoter was replaced by that of the *P. chrysogenum pcbC* gene. There was no *cefD1*, *cefD2*, *cefEF*, or *cefG* transcript signals in samples of untransformed *P. chrysogenum npe6*.

P. chrysogenum Transformants TA64, TA71, and TA98 Have DAC-Acetyltransferase Activity

P. chrysogenum TA64, TA71, and TA98 secreted to the culture broth large amounts of DAC but no cephalosporin C. The conversion of DAC into cephaloslosporin C is catalyzed by the DAC-acetyltranferase encoded by the *cefG* gene. Expression of *cefG* has been described as a limiting step in the production of cephalosporin C in *A. chrysogenum* [27]. To determine if *P. chrysogenum* transformed with the *cefG* gene produces a functional DAC-acetyltransferase, the biochemical activity of this enzyme was determined in extracts of *P. chrysogenum* TA2, TA64, TA71, and TA98. Results showed (Table 1) that the enzymatic activity of DAC-acetyltransferase was present in all these transformants: TA2 (25.8 U/mg protein), TA64 and TA71 (6.7 and 7.2 U/mg protein), and TA98 (30 U/mg protein).

P. chrysogenum Transformants TA64, TA71, and TA98 Synthesize Cephalosporin C Intracellularly that Is Not Secreted

As shown above, *P. chrysogenum* TA64, TA71, and TA98 strains have DAC-acetyltransferase activity. The presence of this enzymatic activity together with the formation of DAC suggested that these strains should be able to synthesize cephalosporin C. Therefore, the intracellular concentration of DAC and cephalosporin C in cells grown in CP medium without phenylacetic acid was studied. Results showed (Figure 5) that *P. chrysogenum* TA71 and TA64 accumulate intracellularly a significant concentration of deacetylcephalosporin C and low amounts of cephalosporin C. *P. chrysogenum* TA98 synthesizes similar concentrations of both DAC and cephalosporin C (Figure 5), in agreement with the higher DAC-acetyltransferase activity found in TA98 than in the two other transformants. These results suggest that the lack of extracellular cephalosporin C in *P. chrysogenum* cannot be explained as the result of low DAC-acetyltransferase activity since cephalosporin C is synthesized intracellularly, particularly in the TA98 transformant (see Discussion).

Table 1. Acetyl-CoA:DAC-Acetyltransferase in the Host Strain *P. chrysogenum* Wis 54-1255 npe6 and in Different Transformants with the *cefD1*, *cefD2*, *cefEF*, and *cefG* Genes

Strain	Genes Added	Acetyl-CoA:DAC-Acetyltransferase Activity (Units/mg of Protein)
P. chrysogenum npe6	none	0
P. chrysogenum TA2	*cefEF*, *cefG*	25.8
P. chrysogenum TA64	*cefD1*, *cefD2*, *cefEF*, *cefG*	6.7
P. chrysogenum TA71	*cefD1*, *cefD2*, *cefEF*, *cefG*	7.2
P. chrysogenum TA98	*cefD1*, *cefD2*, *cefEF*, *cefG*	30.0

Mass Spectra of Accumulated Deacetylcephalosporin C and Cephalosporin C

To confirm the identity of the peaks with retention times of 4.5 and 12.2 min, observed in Figure 5, the mass spectra of the compounds in these peaks was compared with those corresponding to authentic DAC and cephalosporin C. The mass spectra of the 4.5 min peak showed an M^+ ion of 374.17 (Figure 6B) that corresponds exactly to the mass of protonated DAC. The mass spectra of the 12.2 min peak showed an M^+ ion of 416.09 that corresponds exactly to the mass of protonated cephalosporin C. This result confirmed, conclusively, that *P. chrysogenum* is able to synthesize deacetylcephalosporin C and cephalosporin C, although the later compound is not secreted or accumulated extracellularly.

Exogenous Cephalosporin C Is Not Degraded in Culture Broths of *P. chrysogenum* TA98

To test if the extracellular cephalosporin C might be degraded, exogenous cephalosporin C was added at a final concentration of 200 μg/ml at inoculation time to cultures of *P. chrysogenum* TA98 in CP medium. The samples were

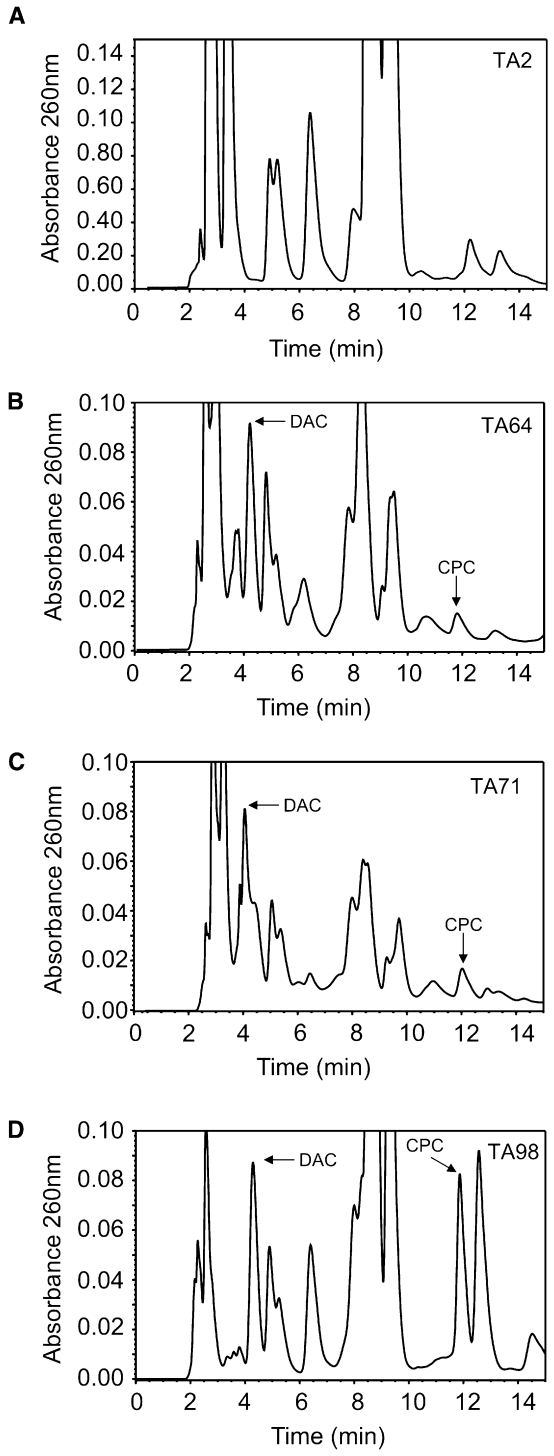

Figure 5. Intracellular Accumulation of Cephalosporin C and the Biosynthetic Intermediates in the Four *P. chrysogenum* Transformants

HPLC chromatograms for determination of DAC and cephalosporin C of crude extracts of (A) TA2 and (B) TA64. TA71 (C) and TA98 (D). All cultures were grown in CP medium without phenylacetic acid for 96 hr.

incubated in a New Brunswick Scientific orbital shaker (250 rpm, 25°C) and quantified by HPLC at 24 hr intervals for up to 96 hr. In a separate experiment, exogenous cephalosporin C was added at 350 μg/ml to culture broths of *P. chrysogenum* TA98 at 72 or 96 hr, and samples were taken and analyzed every 2 hr. Analysis of these samples from the two experiments revealed that the cephalosporin C is not significantly degraded in the culture broths until 96 hr.

Moreover, determination of a possible cephalosporin C acetylhydrolase in culture broths of *P. chrysogenum* TA98 did not show activity in the culture broths by the standard assays described in Experimental Procedures. Taken together, these results indicate that if cephalosporin C is secreted, it would not be degraded since there is no cephalosporin C acetylhydrolase activity (although we can not exclude other degradation system), leaving open the possibility that it is not secreted or it is very inefficiently because of the lack of an adequate cephalosporin transporter (see Discussion).

DISCUSSION

The pathway for penicillin biosynthesis requires the genetic information contained in three genes: *pcbcAB*, *pcbC*, and *penDE* [10, 30, 31]. This cluster is located in a 56.8 kb amplified region [32, 33]. The first two genes (*pcbAB* and *pcbC*) are common to both the penicillin and cephalosporin producing fungi, like *P. chrysogenum*, *P. nalgiovense*, *P. griseofulvum*, *P. verrucosum*, *Aspergillus nidulans* (penicillin producers) [34, 35], and *Acremonium chrysogenum* or *Kallichroma tethys* (cephalosporin producers) [7, 11]. In this work, we have introduced in *P. chrysogenum* the genetic information missing in this filamentous fungus for the biosynthesis of cephem compounds. The work has been facilitated by the previous isolation of a *P. chrysogenum* strain (*P. chrysogenum npe6*) that has an inactive *penDE* gene (encoding a nonfunctional isopenicillin N acyltransferase) [28]. This strain accumulates isopenicillin N; thus, it carries out the first two steps in the biosynthesis of cephalosporins. Introduction of the *cefD1*, *cefD2*, *cefEF*, and *cefG* genes from *A. chrysogenum* conferred to *P. chrysogenum* the genetic information for the epimerization of isopenicillin N into penicillin N, expansion of the thiazolidine ring to the six-membered dihydrothiazine ring, hydroxylation to form deacetylcephalosporin C, and finally the acetylation that produces cephalosporin C.

We observed that recombinant *P. chrysogenum* strains harboring the *cefD1*, *cefD2*, *cefEF*, and *cefG* genes produce a similar amount of β-lactams (the mixture of IPN, penicillin N, and cephalosporins) than the parental strain *P. chrysogenum* Wis 54-1255, i.e., the modified *P. chrysogenum* transformants retain their overall β-lactam biosynthesis ability. Most of the β-lactams produced by *P. chrysogenum* TA64, TA71, and TA98 were penicillinase-resistant cephem compounds, suggesting that *P. chrysogenum* is a good host for heterologous production of cephalosporins, i.e., the IPN that is normally transformed into benzylpenicillin in the wild-type *P. chrysogenum* is

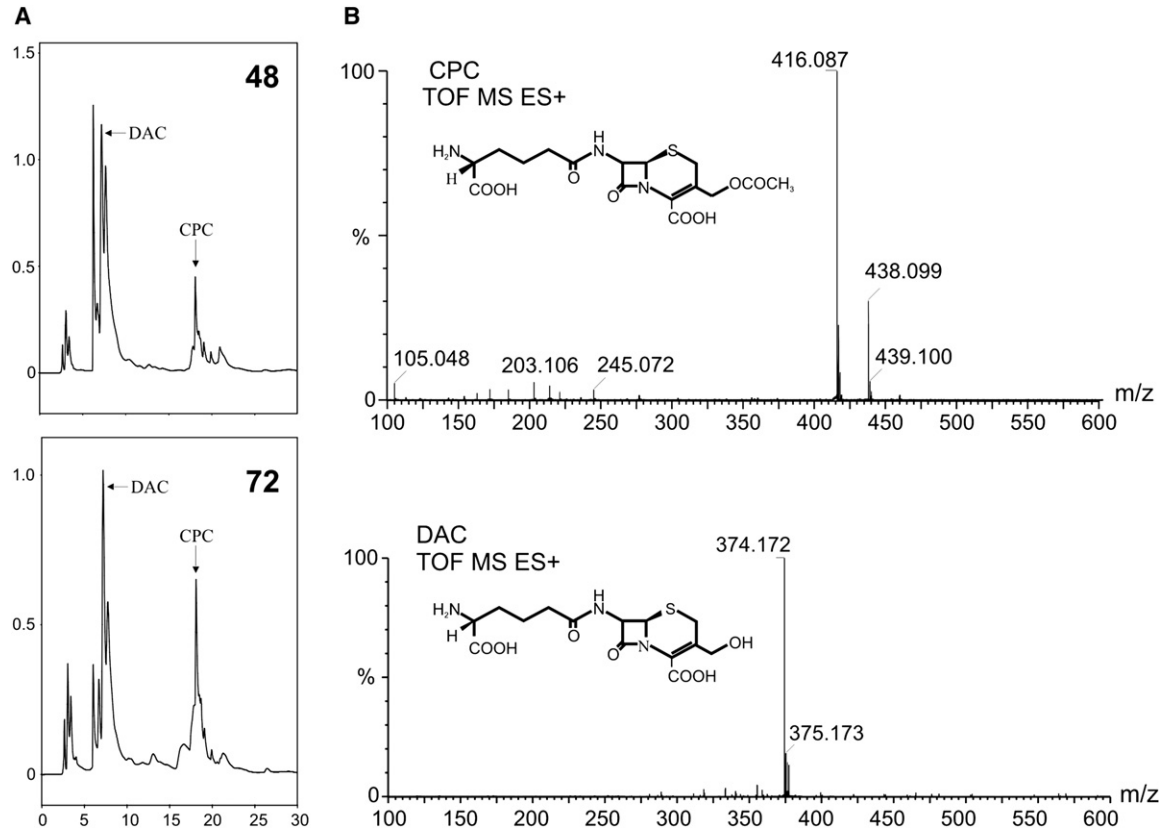

Figure 6. Identification of DAC and Cephalosporin C Accumulated Intracellularly

(A) HPLC chromatograms showing the presence of DAC and cephalosporin C in crude extracts of TA98 grown in DP medium without phenylacetic acid for 48 and 72 hr.

(B) Mass spectra of the DAC and cephalosporin C peaks shown in (A).

converted to cephem compounds in the recombinant strains.

Genetically modified *P. chrysogenum* or *A. chrysogenum* strains for the industrial production of intermediates for semisynthetic β-lactam manufacturing have great interest [36–38]. Cantwell et al. [39] reported a *P. chrysogenum* strain that simultaneously expressed the *cefD* and *cefE* genes from *Streptomyces clavuligerus*; they found only a low production of deacetoxycephalosporin C. In our work, the utilization of the bacterial *cefD* from *Amycolatopsis lactamdurans* (encoding isopenicillin N epimerase) expressed from the *pcbC* promoter combined with the fungal *cefEF* and *cefG* genes also led to recombinant *P. chrysogenum* strains with very poor cephalosporin production, less than 5% of the production obtained with cultures of *P. chrysogenum* TA64, TA71, and TA98 strains that contained the fungal *cefD1-cefD2* genes.

Other recombinant *P. chrysogenum* strains were described by Crawford et al. [40]; these authors obtained *P. chrysogenum* strains transformed with *cefEF* and *cefG* genes from *A. chrysogenum* but lacking the epimerization system. These recombinant strains were reported to produce low levels of adipyl-7-ACA when *P. chrysogenum* fermentations where supplied with adipic acid as side-chain precursor [40]. An explanation for this result is that the ring expansion system might work on adipyl-6-APA without the need of the epimerization system since the adipyl side chain lacks an optically active carbon atom.

Comparative *cefD1*, *cefD2*, *cefEF*, and *cefG* expression studies in *P. chrysogenum* TA98 and *A. chrysogenum* C10 proved that all four genes were expressed in *P. chrysogenum*, including *cefG*. This gene, under the control of the *P. chrysogenum pcbC* promoter, gave a similar transcript level as *cefG* in *A. chrysogenum* C10 from its native promoter.

The *cefD2* gene was expressed in *P. chrysogenum* from its native promoter to a much higher extent than in *A. chrysogenum* C10. This phenomenon is intriguing and is being investigated in more detail. *cefD1* (encoding the isopenicillin N-CoA synthetase) is the cephalosporin gene showing a lower degree of expression in the heterologous host and might be limiting for DAC and cephalosporin C biosynthesis in *P. chrysogenum*. The utilization of the *Acremonium* native *cefD1* promoter may explain this low expression level in the heterologous *P. chrysogenum* host. Promoter replacement by one of the well-known *P. chrysogenum* promoters (e.g., *gpdA*, *gdh*, or *pcbC* [27]) might be used to improve its expression.

The main product of the recombinant strains obtained in this work is deacetylcephalosporin C, a valuable intermediate for the synthesis of chemically derived cephalosporins. Surprisingly, cephalosporin C, the final product of the fungal cephalosporin biosynthetic pathway, is not detected in culture broths. However, cephalosporin C is synthesized and found intracellularly in TA64, TA71, and TA98, as shown by HPLC analysis of cell extracts. Transformants TA64 and TA71 showed low levels of DAC-acetyltransferase activity, what correlates with the higher ratio of intracellular DAC/cephalosporin C observed in these two strains. However, *P. chrysogenum* TA98, which showed a high DAC-acetyltransferase activity, reached an intracellular concentration of cephalosporin C similar to that of DAC.

An important question is why cephalosporin C is not secreted in *P. chrysogenum* TA98 or whether it is secreted and rapidly hydrolyzed by a cephalosporin C-acetylhydrolase. The second hypothesis was ruled out because exogenous-added cephalosporin C remained stable in the CP culture broth under standard cultivation conditions for up to 72 to 96 hr, and no cephalosporin C acetylhydrolase was found in the culture broths of *P. chrysogenum* before this time.

The hypothesis that cephalosporin C is not secreted or it is very inefficiently is plausible since *P. chrysogenum* is not a natural cephalosporin producer, and, therefore, it may not contain a specific transporter for cephalosporin secretion [41]. Indeed, the *cefT* gene that encodes a 12 transmembrane spanning domains MFS protein related to cephalosporin secretion is present in *A. chrysogenum* linked to the early cephalosporin gene cluster [42], but we have not found it in *Penicillium*. If this is the case, the *P. chrysogenum* TA98 would be an excellent host system to clone the gene(s) involved in cephalosporin secretion that may confer to this strain the ability to secrete cephalosporin.

P. chrysogenum is able to produce adipyl-7-ACA a compound more hydrophobic than cephalosporin C [40]. Unfortunately it is not known how this compound is secreted. It might utilize a different MFS protein or an alternative secretion system [41].

SIGNIFICANCE

There have been several attempts to express the missing *cef* genes in *P. chrysogenum* to produce cephalosporin [37, 39]. Using the *S. clavuligerus* epimerase gene, Cantwell et al. [39] found only a low production of deacetoxycephalosporin C in full agreement with our results when we introduced the *cefD* gene from *A. lactamdurans* in *P. chrysogenum* (less than 5% of the production obtained with transformants containing the fungal *cefD1-cefD2* genes). It is important to note that in fungi, epimerization of IPN to PenN is performed by an entirely different enzymatic system that involves activation of IPN to its CoA derivative followed by epimerization of the CoA-activated α-aminoadipic side chain [29]. It is likely that the bacterial epimerase, even when expressed from a fungal promoter (as in our experiments), is not properly targeted in the fungal cells. Therefore, expression of the fungal *cefD1-cefD2* genes in *P. chrysogenum* is a better procedure for obtaining cephalosporin since the two-component fungal epimerase system is properly targeted to the organelles involved in the late steps of cephalosporin biosynthesis. A different approach based on the use of the fungal *cefEF* and *cefG* genes, but lacking the epimerization system, was reported by Crawford et al. [40]. The recombinant strains were reported to produce adipyl-7-ACA when the fermentations were supplied with adipic acid. This procedure appears to rely on the expansion of adipyl-6-APA to adipyl-7-ACA without the involvement of an epimerase system. In conclusion, the expression in *P. chrysogenum* of the fungal *cefD1*, *cefD2*, *cefEF*, and *cefG* genes of *A. chrysogenum* is more adequate than the utilization of the bacterial epimerase system and opens the way for tailored strain improvement. Directed mutation of the bacterial epimerase gene may, however, be also a good method for enhanced IPN epimerization in *P. chrysogenum*.

EXPERIMENTAL PROCEDURES

Strains and Culture Media

Penicillium chrysogenum Wisconsin 54-1255 strain is a low penicillin producing strain containing a single copy of the penicillin gene cluster [32]. *P. chrysogenum npe6 pyrG*, a Wisconsin 54-1255 derivative that lacks acyl-CoA:isopenicillin N acyltransferase [28], was used as host strain for introducing the cephalosporin biosynthesis genes. *Escherichia coli* ESS2231 (β-lactam supersensitive strain) and *Micrococcus luteus* ATCC 9341 were used for routine cephalosporin and penicillin bioassays, respectively. *E. coli* DH5α competent cells were used for plasmid propagation.

P. chrysogenum spores were obtained from plates of Power medium [43] grown for 5 days at 28°C. Seed cultures were initiated by inoculating fresh spores from one Petri dish during 20 hr in CI (Complex Inoculum) medium containing corn steep solids 20 g/l, sucrose 20 g/l, yeast extract 10 g/l, and $CaCO_3$ 5 g/l. Cultures in CP medium (Complex Production), pharmamedia 20 g/l, lactose 50 g/l $(NH_4)_2SO_4$ 4 g/l, and $CaCO_3$ (5 g/l) were inoculated with 5% of 20 hr seed cultures and incubated in a New Brunswick Scientific orbital shaker (250 rpm, 25°C). In some experiments, defined (DP) medium [44] without phenylacetic acid was used. In this case, seed cultures were initiated by inoculating fresh spores in DI (Defined Inoculum) medium [44] and incubated at 25°C for 24 hr; 10% of the seed cultures was used to inoculate cultures in DP medium without phenylacetic acid.

Transformation of *P. chrysogenum* Protoplasts

Protoplasts of *P. chrysogenum* were obtained as described by Fierro et al. [45]. Transformation was performed according to the procedures of Cantoral et al. [46] and Díez et al. [47]. Transformant clones were selected by complementation of the uracil auxotrophy or resistance to phleomycin (30 μg/ml).

DNA Isolation and Southern Blotting

Small amounts of total DNA from *P. chrysogenum* were isolated from mycelium grown in MPPY medium following the protocol described by Casqueiro et al. [48]. Total DNA digested with restriction enzymes were separated by agarose gel electrophoresis and blotted onto nylon membranes (Hybond NX, Amersham Pharmacia Biotech) [49]. For Southern blot analysis, the Dig Easy Hyb system (Roche Diagnostic

Corporation) was used. Hybridizations were performed according to the manufacturer's protocol, and the hybridization signals were visualized with chemiluminescence and recorded on X-ray film with an exposure time of 5 min.

RNA Extraction and Northern Analysis

Total *A. chrysogenum* RNA was isolated with the RNeasy Kit (QIAGEN). Total RNA was resolved by agarose-formaldehyde gel electrophoresis and blotted onto Hybond-N membranes (Amersham Pharmacia Biotech) as described by Sambrook et al. [49].

For northern blot analysis, the Dig Easy Hyb Kit (Roche Diagnostic Corporation) was used according to the manufacturer's protocol. The hybridization signals were visualized with chemiluminescence and recorded on X-ray film with an exposure time of 10 min.

Assay of Acetyl-CoA:DAC-Acetyltransferase

The DAC-acetyltransferase activity (encoded by *cefG*) was assayed in a reaction mixture containing 50 μl of 5 mM acetyl-CoA, 50 μl of 5 mM DAC, 25 μl of 50 mM $MgSO_4$, and cell extract (100 μg of total protein) in 150 μl of 50 mM potassium phosphate buffer (pH 7.0). The mixture was incubated for 1 hr at 37°C. The reaction was stopped by addition of one volume of methanol. After centrifugation, the cephalosporin C formed was analyzed in the supernatant by HPLC. One unit (U) is defined as the activity forming 1 ng of cephalosporin C per min.

Assay of Cephalosporin C Acetylhydrolase Activity

The cephalosporin C acetylhydrolase assay was performed as described by Velasco et al. [50] in cultures of the *P. chrysogenum* transformants in CP medium without phenylacetic acid from 24 hr to 96 hr. The cephalosporin C acetylhydrolase reaction mixture contained cephalosporin C (20 mM) in 50 mM Tris/HCl (pH 8.0) and 75 μl of culture supernatant. The mixture was incubated for 1 hr at 37°C, and the reaction was stopped by the addition of one volume of methanol. The conversion of cephalosporin C to DAC was quantified by HPLC.

HPLC Resolution of Isopenicillin N and Penicillin N

HPLC identification of isopenicillin N and penicillin N was performed by the protocol described by Neuss et al. [51]. Isopenicillin N and penicillin N (500 μg/ml) were derivatized with 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) (2 mg/ml in acetonitrile); the reaction was carried out for 2 hr at pH 8.5 (adjusted with sodium bicarbonate). The HPLC separation was performed in a Beckman System Gold high-pressure liquid chromatograph (HPLC) equipped with a μBondpack C18 column with a mobile phase of methanol-acetonitrile-acetic acid water (36:7:2:55) with a flow of 1.2 ml/min.

HPLC Determination of Cephalosporins: DAC and Cephalosporin C

P. chrysogenum strains were grown for cephalosporin C production in CP medium without phenylacetic acid. Samples of the culture broth were filtered through Whatman filter paper, and the cephalosporin C and DAC were determined in a Beckman System Gold HPLC equipped with a μBondapack C18 column, as described previously by Gutiérrez et al. [27]. The DAC and cephalosporin C analyzed by mass spectrometry were separated by HPLC in a Shimadzu SCL-10AD chromatograph equipped with a SPD-M10A detector and LC-10AD pump by using a reverse-phase μBondapack C18 column. These cephalosporins were eluted with a mixture of solvents A (0.005% formic acid) and B (100% acetonitrile) by using a 0%–50% gradient of solvent B (0% at 0 min, 3% between 2 min and 3 min, and 50% between 18 min and 23 min) with a constant flow of 0.7 ml/min.

Mass Spectrometry

Mass spectra for deacetylcephalosporin C and cephalosporin C were determined by using a LCT-TOF mass electrospray system (Micromass Instruments S.A., Barcelona, Spain) in the electrospray and APCI ionization modes. Samples of pure cephalosporin C and DAC were provided by J.L. Barredo (Antibióticos, S.A.; León, Spain).

Supplemental Data

Supplemental Data include two figures and are available at http://www.chembiol.com/cgi/content/full/14/3/329/DC1/.

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